

Report

Response of *Drosophila* to Wasabi Is Mediated by *painless*, the Fly Homolog of Mammalian TRPA1/ANKTM1

Bader Al-Anzi,¹ W. Daniel Tracey, Jr.,² and Seymour Benzer^{1,*}

¹Division of Biology 156-29

California Institute of Technology

Pasadena, California 91125

²Departments of Anesthesiology, Cell Biology, and Neurobiology

Duke University Medical Center

Durham, North Carolina 27710

Summary

A number of repellent compounds produced by plants elicit a spicy or pungent sensation in mammals [1–16]. In several cases, this has been found to occur through activation of ion channels in the transient receptor potential (TRP) family [1–7]. We report that isothiocyanate (ITC), the pungent ingredient of wasabi, is a repellent to the insect *Drosophila melanogaster*, and that the *painless* gene, previously known to be required for larval nociception, is required for this avoidance behavior. A *painless* reporter gene is expressed in gustatory receptor neurons of the labial palpus, tarsus, and wing anterior margin, but not in olfactory receptor neurons, suggesting a gustatory role. Indeed, *painless* expression overlaps with a variety of gustatory-receptor gene reporters. Some, such as Gr66a, are known to be expressed in neurons that mediate gustatory repulsion [8–10]. *painless* mutants are not taste blind; they show normal aversive gustatory behavior with salt and quinine and attractive responses to sugars and capsaicin. The *painless* gene is an evolutionary homolog of the mammalian “wasabi receptor” TRPA1/ANKTM1 [6], also thought to be involved in nociception. Our results suggest that the stinging sensation of isothiocyanate is caused by activation of an evolutionarily conserved molecular pathway that is also used for nociception.

Results and Discussion

painless Mutant Flies Are Defective in Avoidance of Isothiocyanate

We previously isolated mutations in the *Drosophila* gene *painless* [17], an evolutionary homolog of mammalian isothiocyanates (wasabi) receptor TRPA1/ANKTM1 [18]. We found that the *painless* gene is required in *Drosophila* larvae for activation of sensory neurons by noxious heat, as well as for the behavioral response to noxious thermal and mechanical stimuli [17]. The requirement for *painless* in noxious-heat avoidance appeared to be consistent with the hypothesis that wasabi causes a sensation of burning pain in mammals through activation of the PAINLESS homolog TRPA1/ANKTM1.

This parallel, combined with the known insecticidal properties of isothiocyanate (ITC) [11–16], led us to ask whether *Drosophila* is also repelled by isothiocyanates, and, if so, whether such avoidance behavior is mediated through the product of the *painless* gene.

We used a modified version of the two-choice preference test [19], as shown in Figure 1A. Wild-type Canton-S flies showed aversion to both allyl- and benzyl-isothiocyanate (AITC and BITC, respectively, Figures 1B and 1C). We next examined the avoidance by *painless* mutant flies. Flies mutant for each of three alleles—*pain*¹, *pain*², and *pain*^{GAL4}—showed reduced avoidance of ITC-containing food (Figures 1B and 1C). The degree of avoidance for each allele correlated with the severity of its phenotype observed in nociception assays [17]. *pain*¹ has a P element insertion in the 5′ untranslated region of the *painless* transcript; it is hypomorphic and recessive in mechanical and thermal nociception assays. *pain*², also marked by a P element insertion in the 5′ untranslated region of *painless*, contains a 12 Kb deficiency that deletes promoter and enhancer elements of the *painless* gene. This deficiency results in stronger nociception and isothiocyanate avoidance defects than in *pain*¹ (Figures 1B and 1C). The third allele, *pain*^{GAL4}, contains a GAL4-enhancer trap insertion in the first exon of *painless*; it shows an intermediate isothiocyanate-avoidance defect (Figures 1B and 1C).

To confirm that the isothiocyanate-avoidance defect is actually due to mutation of the *painless* gene, we performed complementation tests among the mutant alleles, as well as transgenic genomic rescue of the *painless* gene. Flies *trans*-heterozygous for *pain*¹ and *pain*² showed a reduced isothiocyanate avoidance similar to that of *pain*¹ and *pain*² homozygous mutants, indicating allelism of the mutations with respect to this phenotype (Figures 1D and 1E). A transgenic construct with a genomic interval containing the *painless* coding region and 2 Kb of upstream DNA—a construct that was previously shown to rescue the nociception defect of *painless* mutant larvae [17]—rescued the isothiocyanate-avoidance defect in adult *painless* mutants (Figures 1B and 1C).

Isothiocyanate Inhibition of the Proboscis Extension Reflex Is Defective in *painless* Mutants

We further examined the taste responsiveness of both wild-type C-S and *painless* mutants toward isothiocyanate by using a proboscis-extension-reflex assay. This assay is based on the observation that, when a hungry fly’s legs encounter a sugary substance, it responds by extending its proboscis. The probability of extension increases with sugar concentration and decreases as the concentrations of aversive-tasting substances mixed with the sugar increase [8–10, 20, 21].

When the legs of wild-type C-S flies were contacted with 1% sucrose, they extended the proboscis in 43% of the trials, on average. However, adding 2 mM AITC or 0.4 mM BITC to the sucrose solution reduced response frequencies to 19% and 18%, respectively, confirming

*Correspondence: benzer@caltech.edu

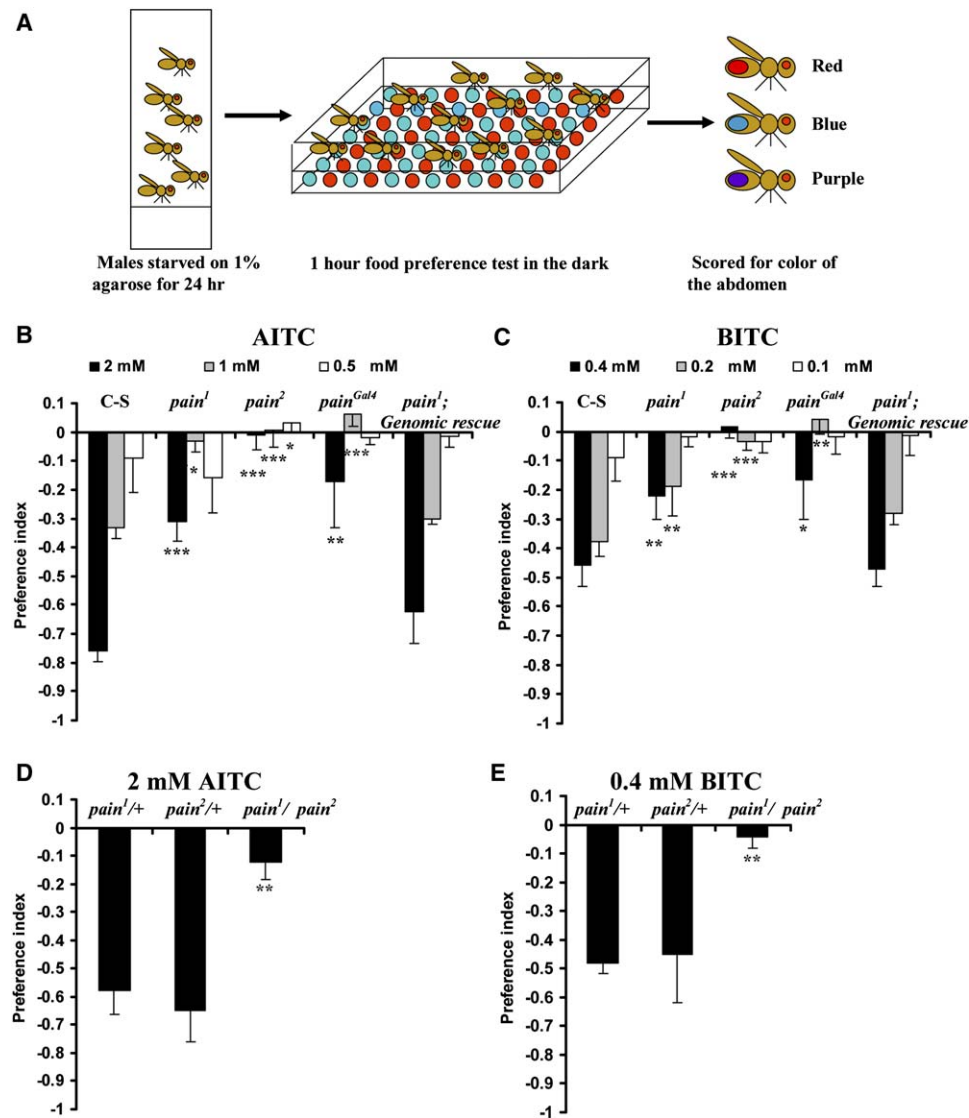


Figure 1. Mutations in the *painless* Gene Cause a Defect in Avoiding Allyl and Benzyl Isothiocyanate

(A) Schematic representation of the two-choice preference test.

(B and C) Wild-type C-S flies avoid 1% sucrose/1% agarose containing either allyl or benzyl isothiocyanate (AITC and BITC, respectively). *painless* mutants (*pain*¹, *pain*², and *pain*^{GAL4}) are defective in this avoidance. This defect is rescued by a transgene expressing a wild-type copy of the *painless* gene.

(D and E) Complementation testing of *pain*¹ and *pain*² confirms that the defect in detecting isothiocyanate maps to the *painless* gene. Each single mutant is recessive, but the *trans*-heterozygotes fail to avoid the isothiocyanates.

Error bars are standard deviation of five experiments for a given tested material concentration as compared to wild-type C-S flies; 80–100 flies per experiment. (Asterisks denote statistical significance: *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.005; Student's *t* test.)

the aversive effect of isothiocyanate on this behavior (Figure 2). The *painless* mutant flies did not show significant AITC or BITC inhibition of sucrose-induced proboscis extension. This defect was rescued in *pain*¹ mutant flies carrying a transgene of wild-type *painless* DNA, which showed near-wild-type levels of AITC and BITC inhibition of the proboscis extension reflex (Figure 2).

All of these results show that the isothiocyanate-avoidance defect seen in *painless* mutant flies is due to loss-of-function mutations in the *painless* gene and that the isothiocyanate-induced aversive response in wild-type *Drosophila* is mediated by the TRPA/PAINLESS TRP channel.

The PAINLESS TRPA Channel Is Expressed in *Drosophila* Gustatory Organs

The feeding responses of hungry flies are initiated by sensory neurons located in the tips of the leg and in the labial palpus [8–10, 19, 20, 22, 23]. To test whether the *painless* gene is expressed in these neurons, we made use of one of our *painless* mutant alleles, *pain*^{GAL4}, which contains a P element carrying a GAL4 insert in the first exon of the gene [17]. To determine whether the enhancer trap expression in *pain*^{GAL4} is relevant to the site of action of the *painless* gene, we used it to rescue the ITC-avoidance defect via expression of a UAS-*painless* transgene on a *painless* mutant background. In the

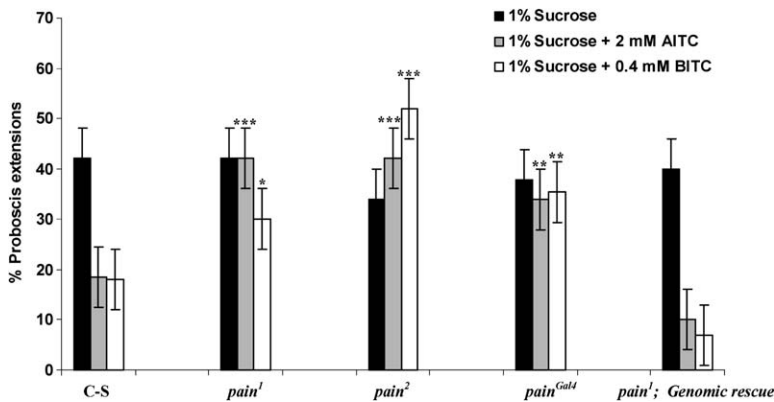


Figure 2. The *painless* Gene Is Required to Produce Isothiocyanate-Induced Inhibition of the Proboscis-Extension Reflex

Hungry, wild-type C-S flies extend the proboscis when their feet are contacted with 1% sucrose. The extension probability decreases when AITC or BITC is added to the sucrose. *painless* mutants (*pain*¹, *pain*², and *pain*^{GAL4}) do not show such a reduction; their response to AITC or BITC in sucrose is similar to sucrose alone. This defect is rescued by a transgene expressing a wild-type copy of the *painless* gene.

Error bars are standard deviations of three experiments, as compared to wild-type C-S flies. N = 30 flies per experiment. (Asterisks denote statistical significance: *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.005; Student's *t* test.)

two-choice preference test, the *pain*^{GAL4} driver did, indeed, rescue the isothiocyanate-avoidance defect observed in *painless* mutants (Figure 3). We therefore mated *pain*^{GAL4} flies to transgenic lines carrying UAS-driven fluorescent reporters, and the resulting progeny were examined for fluorescence by confocal microscopy.

Clear expression was observed in a subset of sensory neurons among gustatory bristles located in the labial palpus (Figure 4B, arrow), the leg tarsus (Figure 4D, arrow), and the anterior wing margin (Figure 4J). All these organs are believed to be involved in detecting gustatory stimuli [8–10, 20, 21, 24]. We further confirmed the identity of those neurons by generating *pain*^{GAL4}; UAS-*dsRed* flies that also carry a GFP reporter expressed in a variety of gustatory neurons in the labial palpus. A significant fraction of *painless*-positive labellar neurons were also positive for the gustatory-neuron reporter Gr66a-GFP (Figure 4C), thus confirming their gustatory nature. Indeed, gustatory neurons expressing Gr66a have been reported to mediate some of the taste-repulsive responses of adult *Drosophila* [8–10]. We also found some overlap between *pain*-GAL4-expressing neurons

and Gr47a-GFP and Gr32a-GFP reporters in the leg (Figures 4E and 4F). The observation that many Gr66a and some Gr32a and Gr47a gustatory neurons express the *painless* reporter suggests the existence of previously undetected ion-channel heterogeneity among gustatory neurons that express the same gustatory receptor. This diversity might contribute to firing specificity of gustatory-neuron response.

We do, consistently, observe *painless*-positive neurons that do not express any of the Gr reporters used, an observation that might indicate that some of the *painless*-positive neurons of the palpus are specifically nociceptive or mechanosensory. However, we have not ruled out the possibility that even those neurons might express gustatory reporters not yet tested for.

Chemoreceptor neurons, like the gustatory neurons, are characterized by dendrites that extend to the tips of the gustatory bristles [21, 24]. *pain*^{GAL4} neurons in the leg and labial palpus do indeed show fluorescent dendrites extending to the bristle tips, further confirming their gustatory nature (Figure 4G, with inset expanded in Figures 4H and 4I, arrows).

The *painless* reporter was undetectable in olfactory receptor neurons of the third antennal segment (Figure 4K, bottom), although some expression was seen in the mechanosensory neurons of Johnston's organ in the second antennal segment, which is not involved in olfactory responses (Figure 4K, arrow). The maxillary palpus also was negative (Figure 4L).

Combined, these expression patterns suggest that the main sensory neurons involved in *painless*-mediated isothiocyanate detection in our assays are largely gustatory, rather than olfactory, in nature.

Within the CNS, CD8-GFP-expressing axons of the *painless* neurons project to the subesophageal ganglion (Figure 4M, with inset expanded in Figure 4N, arrow), where axons that mediate gustatory responses are known to terminate [8–10]. Interestingly, the projection pattern observed for subesophageal expression of *painless*-GAL4-expressing cells is somewhat distinct from previously described projections of cells labeling individual seven-transmembrane gustatory receptors [8–10]. A possible explanation for this unique pattern of *painless*-positive axon projections is that *painless*-positive neurons in the labial palpus include more than one class of gustatory receptor neurons, making their pattern of termination in the subesophageal ganglion

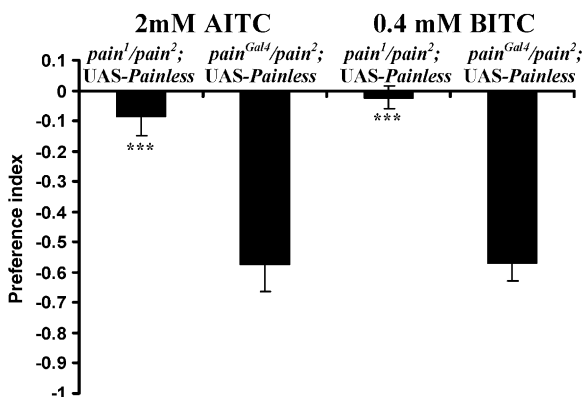


Figure 3. The *pain*^{GAL4} Driver with UAS-*painless* Rescues the Mutant Phenotype

In the two-choice preference test, UAS-*painless* rescues both the AITC and BITC avoidance phenotypes. Error bars are standard deviations of five experiments for a given tested material concentration, as compared to wild-type C-S flies. N = 80–100 flies per experiment. (Asterisks denote statistical significance: *, *p* < 0.05; **, *p* < 0.01; ***, *p* < 0.005; Student's *t* test.)

a broader composite of more than one type of gustatory neuron.

In addition, strong CD8-GFP expression was observed in the mushroom body (Figure 4M, arrow), as well as in cells in various regions of the brain. GFP-positive axonal tracts from the legs were observed to project into the ventral ganglion (Figure 4O, arrow), which also contains a posterior cluster of cells that are GFP positive (Figure 4O, arrowhead). The projection of some *painless*-expressing fibers to the abdominal ganglion is not surprising, given that the *painless* gene is also involved in detecting various noxious stimuli, including heat and mechanical stress [17].

The Failure of *painless* Mutants to Avoid Isothiocyanate Is Not Due to a General Gustatory Defect

Given the expression pattern of *pain*^{GAL4} in sensory neurons of gustatory organs, one possible explanation for the inability of *painless* mutant flies to detect isothiocyanates could be that *painless* mutant flies are “taste blind.” To address this issue, we tested the ability of *painless* mutant flies to avoid quinine and NaCl, which are known to be gustatory repellents for *Drosophila* [8–10, 20, 21, 24]. When tested in our paradigm, *painless* mutant flies showed normal avoidance of these two compounds (Figures 5A and 5B). We also tested the ability of *painless* mutants to be attracted to sucrose, glucose, and fructose (in 1% agarose, as compared to 1% agarose alone). The attractive response to these sugars was also normal (Figures 5C, 5D, and 5E). These results indicate that the product of the *painless* gene is not required for gustation, per se, because the mutants, although defective in avoidance of isothiocyanate, show normal avoidance of salt and quinine and normal attraction to sugars.

Both Wild-Type and *painless* Mutant Flies Show Positive Preference for Capsaicin

Capsaicin and isothiocyanate both induce burning sensations in mammals, yet the two compounds target distinct TRP channels: TRPA1/ANKTM1 for isothiocyanate and TRPV1 for capsaicin [1, 7]. The burning sensations produced by both wasabi and capsaicin may arise from the fact that the expression patterns of TRPA1 and TRPV1/ANKTM1 overlap in nociceptive neurons [1, 7]. To determine whether capsaicin, like isothiocyanate, is also aversive to flies, we used the same two-choice assay. In contrast to isothiocyanate, wild-type flies, given a choice between capsaicin-laced sucrose and sucrose alone, showed a preference for capsaicin at all three concentrations tested (Figure 5F). *painless* mutants also showed the same positive preference for capsaicin (Figure 5F). This suggests that capsaicin detection in flies does not involve the PAINLESS channel. It is interesting to note that, in a recent study using an assay that counts the number of flies that preferentially roam on agarose mixed with capsaicin versus plain agarose, wild-type flies failed to show any preference [9]. Two major differences between that test and ours could explain the different results. Our assay was capsaicin plus sucrose versus sucrose alone, and the preference was measured by actual ingestion of the food. It will be interesting to further explore the possible subtle interactions involved. The

different actions of isothiocyanate and capsaicin in flies is not surprising, because capsaicin is thought to play a specific role in deterring mammalian herbivores [25, 26]. Although birds, mammals, flies, and nematodes each contain TRP channels in the capsaicin receptor family (TRPV) only the mammalian channel contains a binding site for capsaicin. Thus, the ability of flies to detect capsaicin is unlikely to be mediated by direct binding to a TRP channel. Rather, activation of seven-transmembrane gustatory receptor proteins is likely to be involved.

Conclusions

We have shown that the *painless* gene is required for avoidance of isothiocyanate, the primary pungent ingredient of wasabi and mustard oils. *painless* mutants are defective in avoiding isothiocyanate-spiked food, without being taste blind. Indeed, they show normal preference to sugars and capsaicin and normal avoidance to NaCl and quinine. The pattern of expression of the *painless* gene points to the involvement of gustatory repulsive neurons, rather than olfactory neurons, in the isothiocyanate avoidance response. Nevertheless, we cannot exclude the possibility that wasabi evokes a nociceptive (stinging) response in those neurons, as it does in our own. Given the proposed function of the mammalian homolog of the PAINLESS channel, the TRPA1/ANKTM1 channel, as a mediator of the wasabi response [4–6], our evidence suggests that the molecular mechanisms of detecting and avoiding plant isothiocyanate toxin are conserved between insects and ourselves.

Our work also indicates that the role of TRPA1/ANKTM1/PAINLESS TRP channels in pain detection may have existed before the divergence of vertebrates and insects from their common evolutionary ancestor more than 700 million years ago. This further demonstrates the relevance of *Drosophila* as a model for understanding the evolutionarily conserved aspects, and possibly the core components, of the pain response.

A study recently appeared [27] reporting on a mouse knockout mutant of TRPA1 that bears interesting similarities to the *Drosophila* *painless* mutant, although food ingestion was not involved. A genetic dissection approach to the isolation of other mutants insensitive to wasabi may therefore identify other players in this important, conserved signaling pathway.

Experimental Procedures

Genetics and Fly Strains

Drosophila stocks were raised on standard Caltech propionic acid fly food medium at 25°C on a 12-hr light/dark cycle. All experiments were performed with 4–5-day-old adult males, raised in groups of 20 per vial.

Gr-GFP reporter flies were kindly donated by Kristin Scott and Hubert Amrein.

UAS-*painless* flies were generated by using the polymerase chain reaction (PCR) to amplify the coding region of *painless* from BAC R08 I14 and cloning the product into the PCRXL vector (Invitrogen). The insertion was then cloned into Asp718I NotI-digested pUAST transformation vector, and transgenic flies were produced by standard techniques, with the assistance of Alice Robie. The coding region of UAS-*painless* contains a single nucleotide difference from the published reference sequence that results in a coding change (S358 to R). For the rescue experiment, an insertion on the third

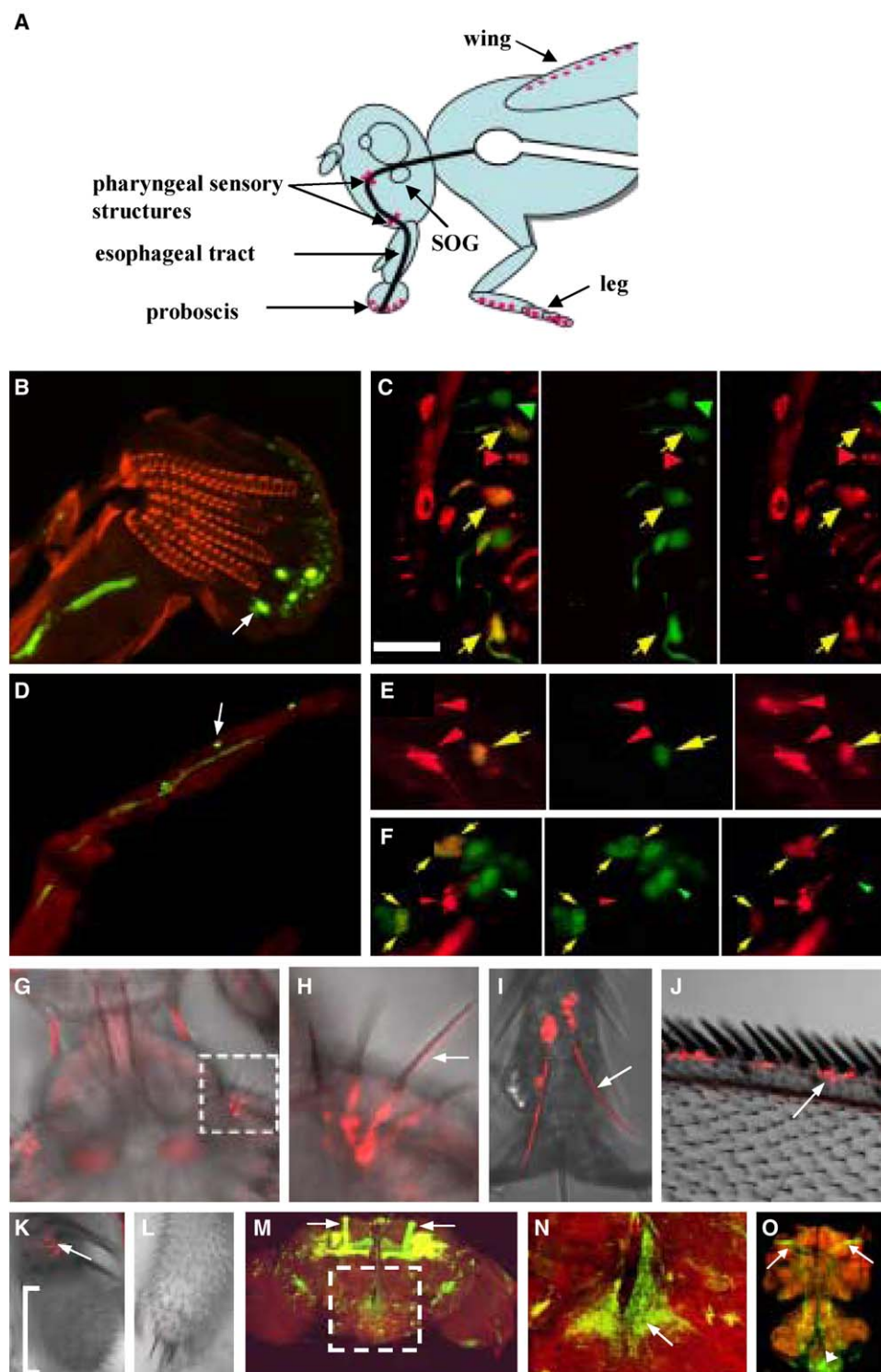


Figure 4. With CD8-GFP or dsRed as a Marker, *pain^{GAL4}* Is Seen to Be Expressed in Gustatory Receptor Neurons of the Proboscis, Internal Pharyngeal Sensory Structures, the Legs, and the Wings

(A) Cartoon of gustatory-neuron sites in the adult fly (red circles).

(B) *pain^{GAL4}* expression in sensory neurons of the labial palpus.

(C) In the labial palpus, seen at higher magnification, a subset of the *pain^{GAL4}*-expressing neurons also express a reporter for the gustatory receptor Gr66a. The red channel (right panel) shows UAS-dsRed driven by *pain^{GAL4}*, and the green channel (middle panel) shows GFP under direct control of the Gr66a promoter (Gr66aGFP). In the merged image (left panel), GRNs that express both *painless-GAL4* and Gr66a-GFP are marked by yellow arrows. Some *painless-GAL4*-expressing neurons did not express Gr66a (red arrowheads). Conversely, some neurons expressed Gr66a-GFP, but not *painless-GAL4* (green arrowheads).

(D) *pain^{GAL4}* is expressed in some of the sensory neurons in the leg (arrow). A subset of the *painless*-positive leg neurons colocalize with the Gr-GFP reporter, confirming their gustatory nature.

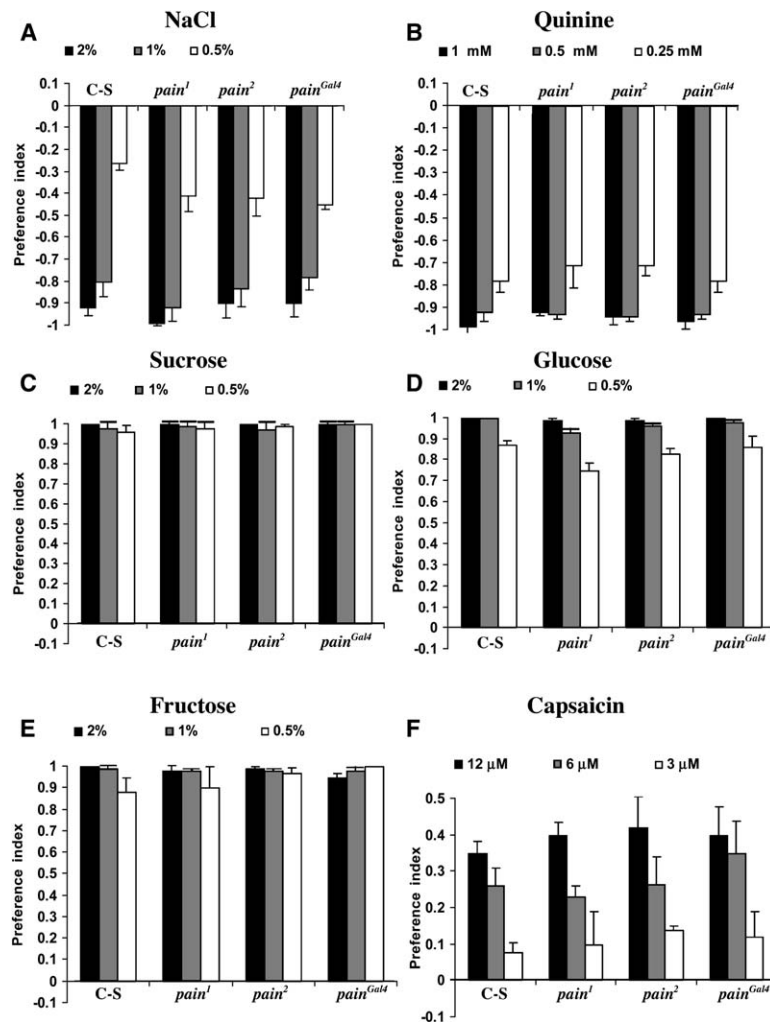


Figure 5. *painless* Mutants Do Not Have a General Gustatory Defect

Both wild-type C-S and *painless* mutant flies show similarly high avoidance of (A) NaCl or (B) quinine. Given a choice between plain 1% agarose versus 1% plain agarose plus sucrose, glucose, or fructose, the flies showed normal preference for all three sugars (C–E). (F) Both wild-type C-S and *painless* mutant flies show similar preference for capsaicin in sucrose versus sucrose alone (all in 1% agarose). Error bars are standard deviations of five experiments for a given tested material concentration, as compared to wild-type C-S flies. N = 80–100 flies per experiment.

chromosome was used to create a stock with *pain*^{GAL4};UAS-*painless*^{AR9}.

The Two-Dye Food Preference Test

We used a modified version of the two-choice preference test [19]. In each run, we used a 96-well microtiter plate that provided two food choices pipetted in the alternating well rows. One food choice was 1% agarose/1% sucrose mixed with either AITC or BITC in 75% ethanol. The other was 1% agarose/1% sucrose with only the ethanol carrier. The food dyes used to label the different food types on each plate were FD&C Blue No. 1 (0.125 mg/ml) and FD&C Red No. 3 (0.5 mg/ml) (Calico Food Ingredients). In each run, 80–100

4-day-old male flies, previously starved for 24 hr, were allowed to choose between 1% sucrose (in 1% agarose) with and 1% sucrose (in 1% agarose) without allyl or benzyl-isothiocyanate (AITC and BITC, respectively). For enhancing the random sampling of the wells by the flies, the distance between plate lid and the surface of the food was limited to 1.5 cm (Figure 1A). The flies were allowed to feed for 1 hr at 25°C, the test being performed in darkness to ensure that the color of the food did not influence choice. At the end of the test period, the flies were frozen, and their abdomens were scored for red, blue, purple, or lack of color. In each experiment, the red and blue dyes were interchanged, and the results were combined. Preference index was defined as the fraction of flies with abdomens

(E and F) The red channel shows UAS-dsRed expression driven by *pain*^{GAL4} (right panels); the green channel shows GFP expression under Gr47a and Gr32a (middle panels). GRNs that express both *pain*^{GAL4} and Gr reporters are marked by yellow arrows. Some *pain*^{GAL4} neurons did not express either Gr47a-GFP or Gr32a-GFP (red arrow, [E] and [F], respectively), and some Gr47a-GFP neurons did not express *pain*^{GAL4} ([F], green arrows). Note that the dsRed protein sometimes forms aggregates and punctate fluorescence. *pain*^{GAL4}; UAS-dsRed-expressing cells extended their dendrites to the bristle-like shaft, as is typical of chemosensory neurons.

(G and H) (G) shows the dsRed signal in dendrites extending to the bristle shaft in the labellum ([H] shows enlarged view of inset in [G]).

(I) Similar expression in tarsal bristles.

(J) *pain*^{GAL4} expression in sensory neurons of the wing margin.

(K) No dsRed expression is seen in the olfactory neurons of the third antennal segment, but some expression is seen in the mechanosensory Johnston's organ of the second antennal segment (arrowhead).

(L) The maxillary palpus is also negative.

(M) With UAS-mCD8-GFP as a marker, strong *pain*^{GAL4} expression is observed in the mushroom body ([M], arrow), as well as in various cell bodies. The axons of some *pain*^{GAL4}-positive sensory neurons terminate in the subesophageal ganglion in areas surrounding the esophagus (as shown in the expanded inset ([N], arrow).

(O) GFP expression is also seen in axonal tracts projecting from the leg to the thoracic ganglion, as well as in a cluster of cells at the posterior end of the ganglion (arrowheads).

showing the food color added to the test substance, minus the fraction of flies showing the alternative color. A preference index close to +1 indicated that the flies were attracted to the tested material, whereas -1 indicated strong rejection. Lack of color indicated failure to feed during the test period, which typically occurred in 5%–10% of the flies; those flies were not included in the calculations. Flies with purple abdomens, having ingested both red and blue food, were assigned half to each group.

Proboscis-Extension Assay

Four-day-old male flies, previously starved for 24 hr on 1% agarose, were anaesthetized by chilling on ice. They were then glued by their backs to a glass slide and allowed to recover for 2 hr at room temperature. Flies that showed no sign of movement after the recovery period were discarded. The remaining flies were given water on a cotton swab until satiation and were then used for the proboscis-extension assay. In this assay, each fly was briefly touched for 5 s on the legs with a cotton swab soaked in the test solution, and the presence or absence of extension was recorded. This stimulus was repeated five times, with a 2 min rest period between repetitions.

Microscopy

The labial palpus, brain, or leg of a male fly *pain^{GAL4}*; UAS-CD8::GFP or *pain^{GAL4}*; UAS-dsRed/Gr-GFP was dissected and mounted beneath a coverslip in PBS. The specimen was then imaged under a 63X 1.NA oil immersion lens on an inverted Zeiss LSM 5 Live confocal microscope, or on an upright Zeiss LSM 510 microscope. The red channel (excitation λ : 532 nm or 568 nm; emission λ : band pass 560–635 nm) and green channel (excitation λ : 488 nm; emission λ : band pass 500–525 nm) were acquired in multitrack mode, zoom factor 1 (pixel dimension 0.1 μ m \times 0.1 μ m \times 0.4 μ m). The acquired Z stack was flattened as a maximum-intensity projection and exported to a TIFF file. Cropping, adjustment of brightness and contrast, and addition of arrows were performed in Adobe Photoshop.

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